PHARMACOLOGY #467C

Comm.
Dr. Bing
Dr. Gardner
Dr. Jacobson

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

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Date: February 1, 1973

1. Name of Investigator(s): (Include Title and Degrees)

2. Institution & Address:

Thomas C. Westfall, A.B., M.S., Ph.D.
Associate Professor of Pharmacology
Department of Pharmacology
University of Virginia School of Medicine
Charlottesville, Virginia

3. Short Title of Project: Action of Nicotine on Peripheral and Central Neurons In Animals Chronically Exposed to Nicotine.

4. Proposed Starting Date: March 1, 1973

5. Anticipated Duration of this Specific Study: Two Years

6. Brief Descripton of Objectives or Specific Aims:

The main objective of this study is to compare the effect of nicotine on several parameters of neuronal activity when administered to naive preparations (tissues obtained from animals not previously exposed to nicotine) or tissues obtained from animals which have been constantly exposed to nicotine for varying lengths of time. The parameters to be measured are: a) release of norepinephrine from peripheral adrenergic neurons (perfused heart preparation), b) release of norepinephrine, dopamine or serotonin from central neurons (perfused brain slice preparation), c) turnover of norepinephrine, dopamine or serotonin and d) monoamine oxidase activity and catechol-O-methyl transferase activity. The study is based on the fact that we have very reliable and reproducible methods for measuring these effects and that smokers are constant users of tobacco. By comparing the effect of nicotine on tissues obtained from animals which have not been previously exposed to nicotine with those that have been exposed for varying periods of time we should have a more valid means of correlating the effect of nicotine on the nervous system in smokers and non-smokers.

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7. Give a Brief Statement of your Working Hypothesis:

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7. Give a Brief Statement of your Working Hypothesis:

A) That measurements of the effect of nicotine on neuronal function can best be determined by comparing the effect on tissues not previously exposed to nicotine with those that have been exposed to nicotine for varying lengths of time. Measurements made on the latter tissue will more closely mimic or correlate with what might be expected in chronic smokers.

B) A second hypothesis is that there may be marked differences in the behavior of neuronal tissue to nicotine when these tissues are taken from animals that have been chronically exposed to nicotine.

8. Details of Experimental Design and Procedures:

A) PRESENT STATE OF KNOWLEDGE IN THE FIELD AND PREVIOUS WORK DONE ON THIS PROJECT.

Nicotine, an important pharmacological ingredient of tobacco, is known to have marked effects on the nervous system. It stimulates autonomic ganglia, the adrenal medulla, the skeletal-neuro muscular junction, certain sensory nerve endings, and has effects in the central nervous system (1-3). In addition, there is also convincing evidence that nicotine produces pharmacological effects by releasing norepinephrine from adrenergic nerve terminals. For instance, this latter effect is seen in preparations devoid of sympathetic ganglia (4,5) and is reduced by procedures which interfere with the functional integrety of the adrenergic nervous system including reserpine (6-9), 6 hydroxydopamine (10), adrenergic blocking agents (4,9,11) and denervation (12).

Over the last couple of years we have been investigating the effect of nicotine on adrenergic nerve terminals using the isolated perfused guinea-pig heart, prelabeled with ^{3}H-norepinephrine as a model (See data submitted with application of August, 1971 and the enclosed reprint (13). We have observed that nicotine produces an explosive increase in the efflux of ^{3}H-norepinephrine (release) from the perfused heart and it is the released amine which produces the sympathomimetic pharmacological effects (positive inotropic and chronotropic activity). There is strong evidence that the mechanism of this action is due to activation of a receptor (13) (see enclosed reprint) located on the axonal membrane of the adrenergic nerve plexus. The effect has an absolute requirement for extracellular Ca^{++}, and can be selectively blocked by pharmacological agents which will not block the release of norepinephrine by other drugs such as tyramine, KCl and aminophylline (Fig. 1,2; Table 1) (13).

Recently we have been able to demonstrate a release of norepinephrine from chopped brain tissue incubated with labeled norepinephrine (Fig. 3) as well as superfused brain tissue (Fig. 4,5,6). It is thought that many of the effects of nicotine on the central nervous system are due to the release of norepinephrine and other neurotransmitters.

A release of ^{3}H-NE has so far been observed from the rat hypothalamus, cortex, medulla-pons and cerebellum (Fig. 3,4). The effect is dependent upon extracellular Ca^{+2} and the release is blocked by hexamethonium and acetylcholine (Fig. 5).

Althought these studies are quite important in defining the biochemical and molecular mechanism of action of nicotine, a question of paramount importance is: TO WHAT EXTENT DOES THE ACUTE ADMINISTRATION

IN VIVO OR IN VITRO REFLECT THE ADMINISTRATION OF NICOTINE IN HUMANS IN THE FORM OF TOBACCO SMOKING? It would appear that studies carried out after animals were chronically exposed to nicotine would come much closer in mimicing the human situation. There are in fact several observations from our own laboratory indicating differences between the acute and chronic administration of nicotine on adrenergic neuronal activity:

1) Injections of nicotine in divided daily doses results in an increase in the 24 hour urinary excretion of catecholamines but after 14 days of continued nicotine administration the elevated urinary catecholamine levels are normal. A study of the mechanism of the return to normal of the elevated urinary catecholamines after chronic administration revealed that there was a significant increase in the monoamine oxidase activity of the heart and liver and an increase in the catechol-o-methyl transferase activity of the liver. It was concluded that tolerance to nicotine induced elevations of urinary catecholamines was due to increased metabolic enzyme activity resulting in faster metabolism of the catecholamines released from the adrenal medulla and adrenergic nerve terminals (see the enclosed manuscript Biochem. Pharmacol. 20: 1627, 1971 (14).

2) Acute injections of nicotine had no effect on the turnover of norepinephrine (a marker for adrenergic nerve activity) in the rat heart but following chronic daily administration for 47 days there was a significant increase in amine turnover (15) (see the enclosed manuscript Eur. J. Pharmacol. 10: 19, 1970). The mechanism of this difference between the behavior on norepinephrine turnover before and after chronic exposure to nicotine is not clear but certainly warrents further investigation.

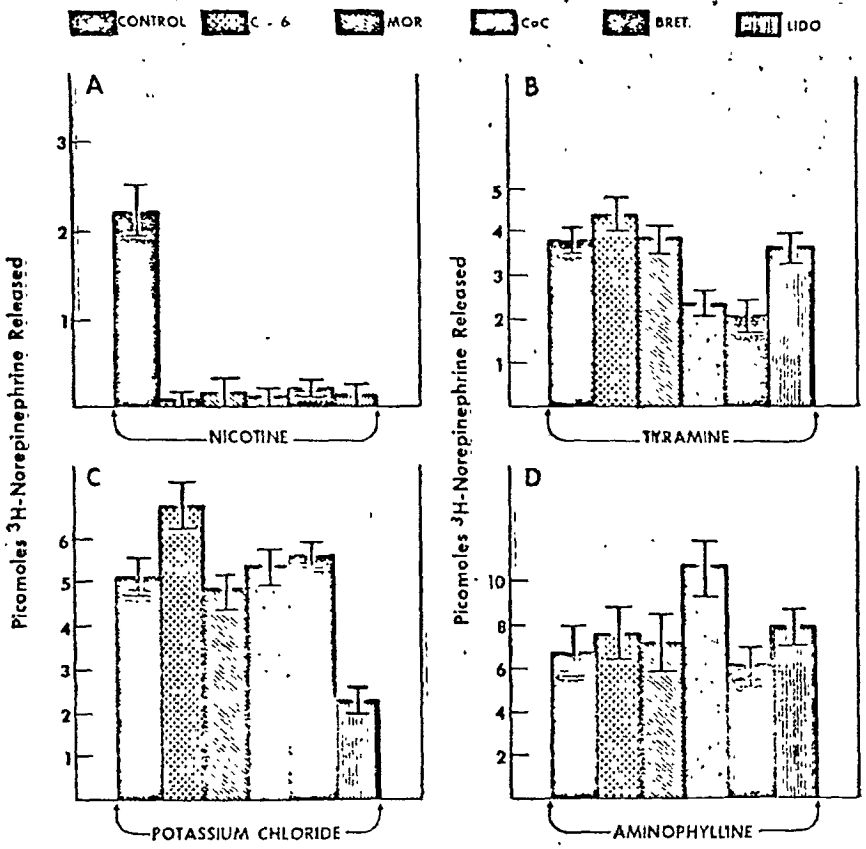

Fig. 1 This figure depicts the effect of nicotine 100µg (panel A), tyramine 300µg (panel B), potassium chloride .3M (panel C) and aminophylline 50mg (panel D) on the release of ^{3}H-norepinephrine from the perfused guinea-pig heart alone or in the presence of hexamethonium (C-6 10^{-5}M), morphine $3X10^{-4}$, cocaine 10^{-5}M, bretylium 10^{-5}M and lidocaine $5X10^{-5}$ ± S.E.M. I. It can be seen that all 5 drugs blocked the release of ^{3}H-NE to nicotine, while only cocaine and bretylium reduced the release by tyramine and lidocaine, the release by ^{3}H-NE by KCl. None of the drugs blocked the release by ^{3}H-NE aminophylline.

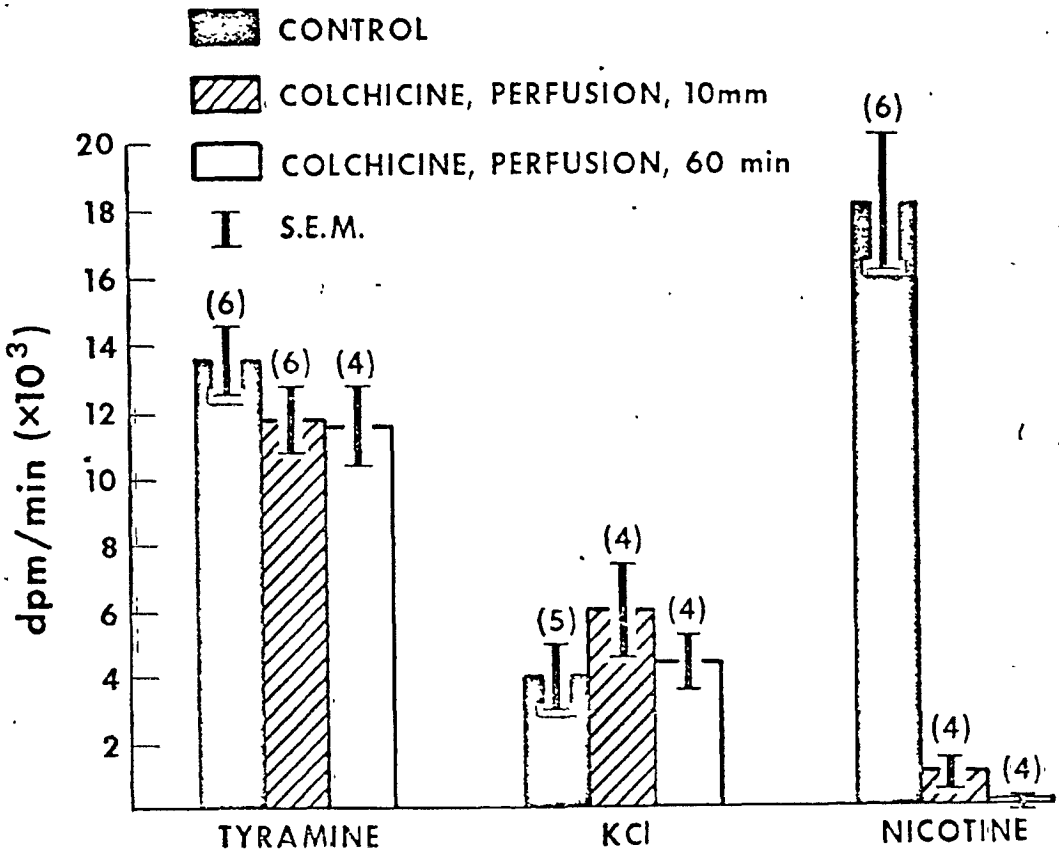

Fig. 2 Shows the effect of tyramine, KCl and nicotine on the release of ^{3}H-norepinephrine from the perfused guinea-pig heart alone or in the presence of colchicine. Colchicine was perfused for 10 min ($5X10^{-5}$M) for 60 min ($5X10^{-5}$M) prior to injecting tyramine, KCl or nicotine. Data is plotted as peak release in dpm/min $X10^{-3} \pm$ S.E. M. (I). Numbers above the bars represents number of experiments. It can be seen that colchicine perfused for either 10 or 60 min did not alter the release of ^{3}H-NE by tyramine or KCl but significantly inhibited the release produced by nicotine.

Table 1: EFFECT OF PROSTAGLANDINS ON THE RELEASE OF ^{3}H-NOREPINEPHRINE BY VARIOUS AGENTS IN THE PERFUSED GUINEA-PIG HEART

Releasing Agent	Control	PGE_1	PGE_2	$PGF_{2\alpha}$
	Δ in ^{3}H-Norepinephrine dpm/min.+ S.E.M.			
Nicotine (100 µg)	16,000 ± 1,500	10,500* ± 1,121	7,200** ± 1,500	5,110** ± 995
KCl (.3M)	49,071 ± 6,000	37,025* ± 4,953	32,489** ± 3,211	36,985* ± 2,437
Tyramine (300 µg)	11,599 1,200	9,092 1,560	16,264* ± 102	18,516** ± 820
Aminophylline (50 mg)	40,023 ± 5,498	61,360** 2,195	62,514* 10,000	51,178* 5,027

*P < .01

**P < .001

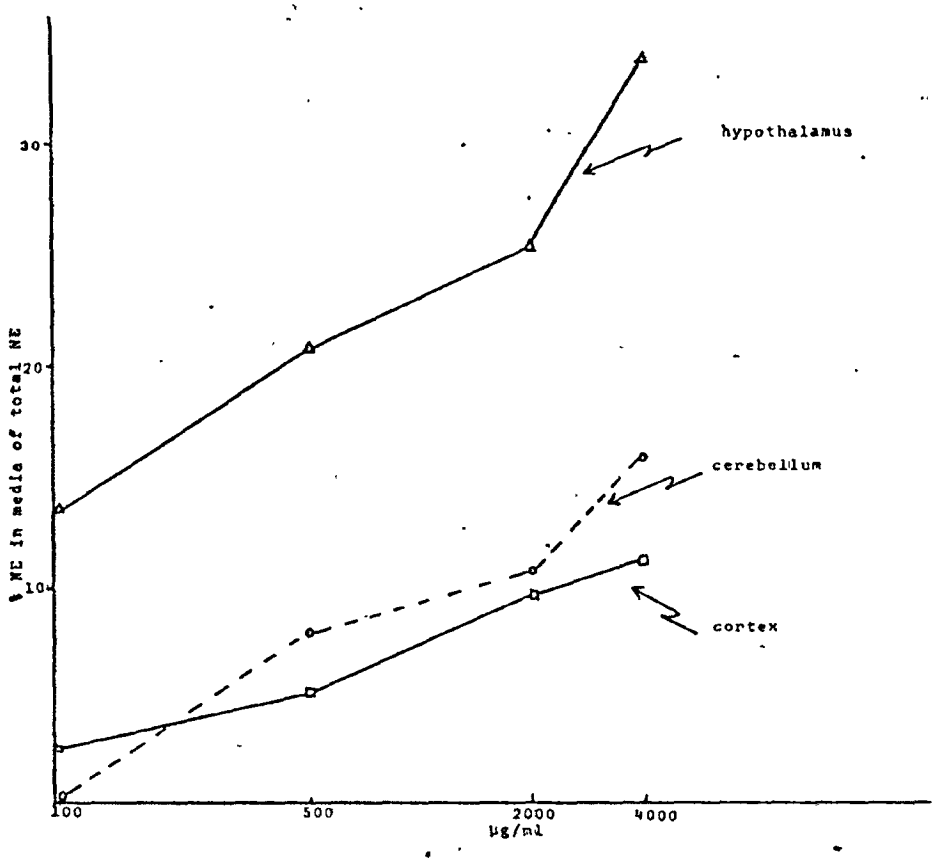

Fig. 3 Depicts dose response curves plotting the effect of nicotine on the release of ^{3}H-NE from incubated chopped brain slices from 3 different brain regions. Conc. of nicotine in µg/ml is plotted on abcissia and % NE in the media as % of total NE on the ordinate. It can be seen that there is a dose related release of ^{3}H-NE from all three brain regions with the release being greatest from hypothalamus.

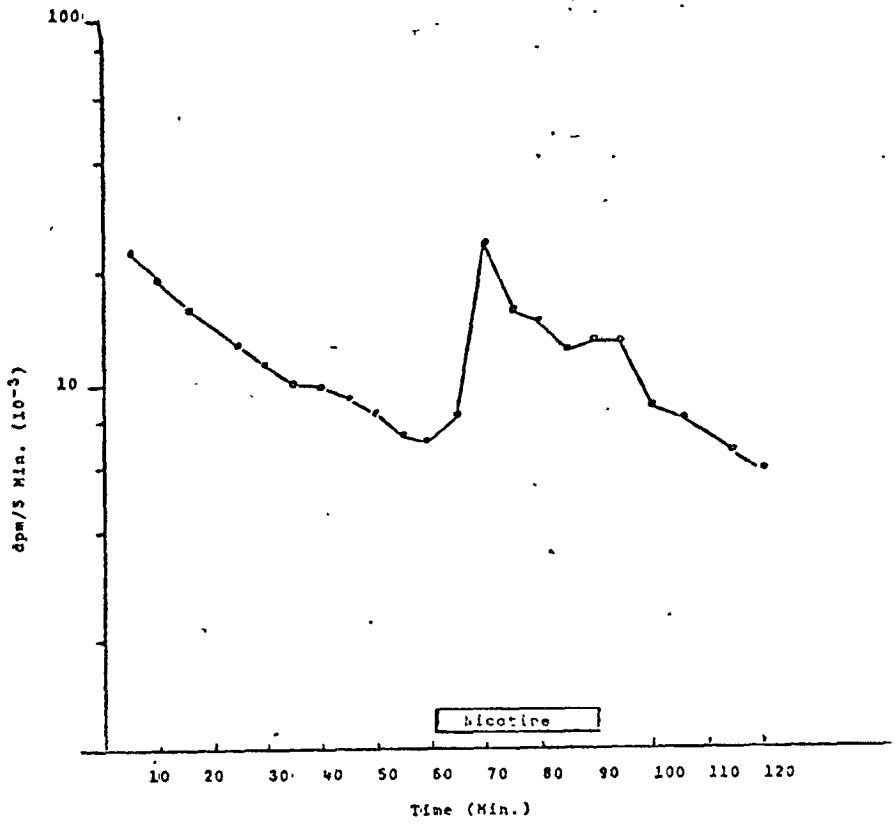

Fig. 4 Depicts the efflux of ^{3}H-NE from the superfused medulla-pons of the rat. The medulla-pons was chopped into .3mm slices in two directions, incubated with ^{3}H-NE, washed and layered on Whatman No. 1 filter paper, placed in a Millipore Filter holder. The chopped slices were then superfused with Krebs-Henseleit solution at a constant flow of 0.6 ml/min and the perfusate continuously collected and analyzed for ^{3}H-NE. Data is plotted as dpm/ 5 min (10^{-3}) against time in min. Following 60 min. Nicotine in a conc. of 1mM was added to the perfusion solution for 30 min. It can be seen that the addition of Nicotine produced a marked increase in the release of ^{3}H-NE.

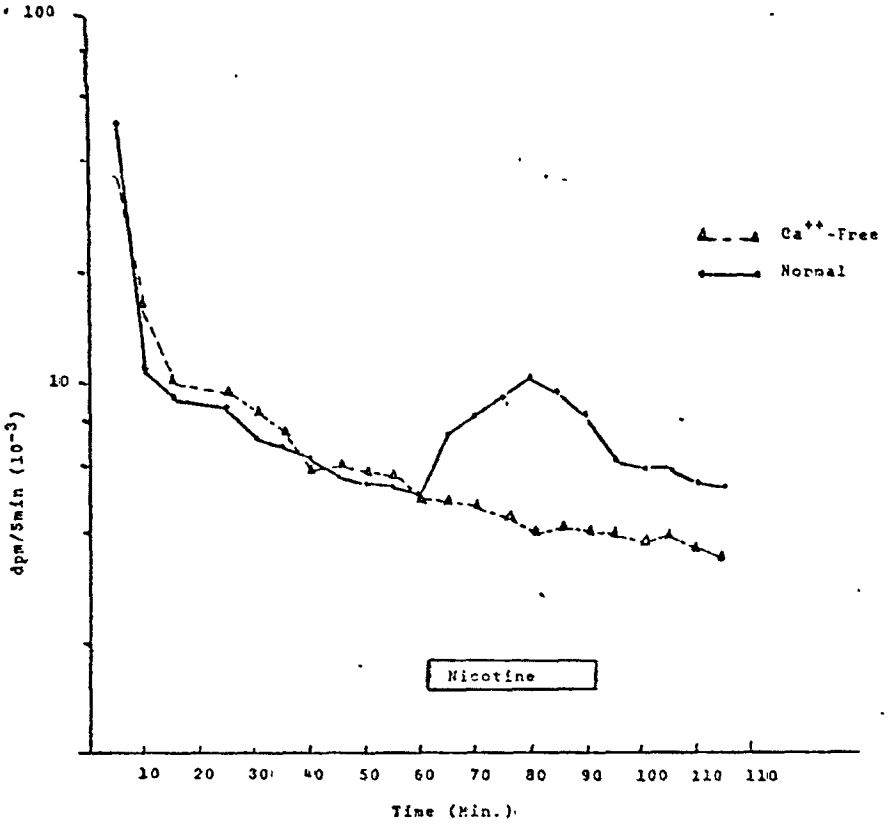

Fig. 5 Depicts the efflux of ^{3}H-NE from the superfused hypothalamus of the rat. The hypothalamus was dissected out and prepared for superfusion according to the procedure described for the medulla-pons in Fig. 4. Data is plotted in a similar fashion as dpm/ 5 min (10^{-3}) vs time in min. The solid curve shows the effect of nicotine in slices perfused with normal medium, the dotted curve depicts slices perfused with a solution devoid of Ca^{++} It can be seen that nicotine produces an increase in the release of ^{3}H-NE. Removal of Ca^{++} from the perfusion solution completely blocks the nicotine induced release of ^{3}H-NE.

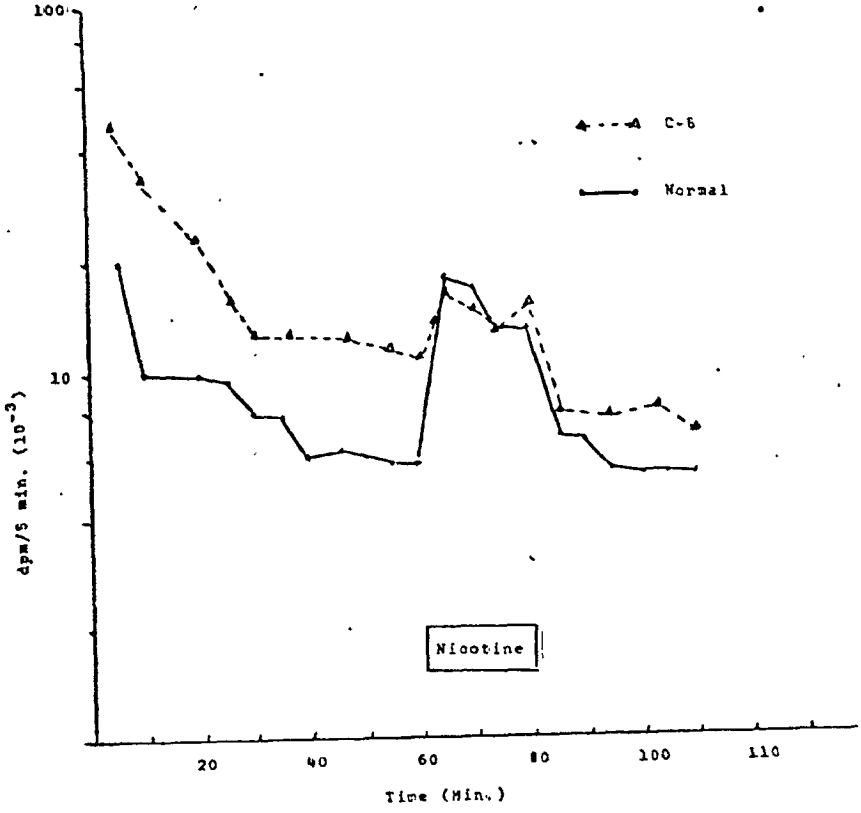

Fig. 6 Depicts the efflux of ^{3}H-NE from the superfused hypothalamus of the rat. The tissue was prepared in a similar fashion as in Fig. 4 and 5. The solid curve shows the effect of nicotine alone while the dotted curve shows the effect of nicotine in the presence of the ganglion blocking agent, hexamethonium (C-6). It can be seen that the presence of hexamethonium markedly reduces the release of ^{3}H-NE produced by nicotine.

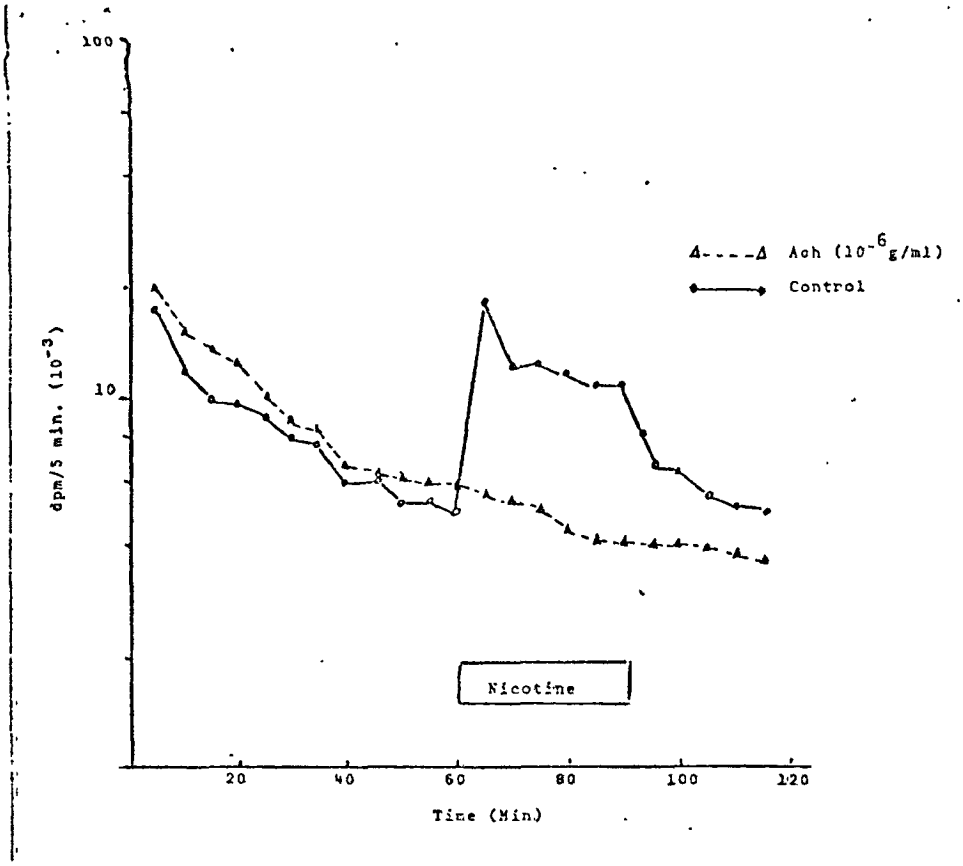

Fig. 7 Depicts the efflux of ^{3}H-NE from the superfused hypothalamus of the rat. The tissue was prepared in a similar fashion as in fig. 4, 5 and 6. The solid curve shows the effect of nicotine alone while the dotted curve shows the effect of nicotine in the presence of acetylcholine (10^{-6}g/ml). It can be seen that in the presence of this conc. of acetylcholine the effect of nicotine in releasing NE is blocked.

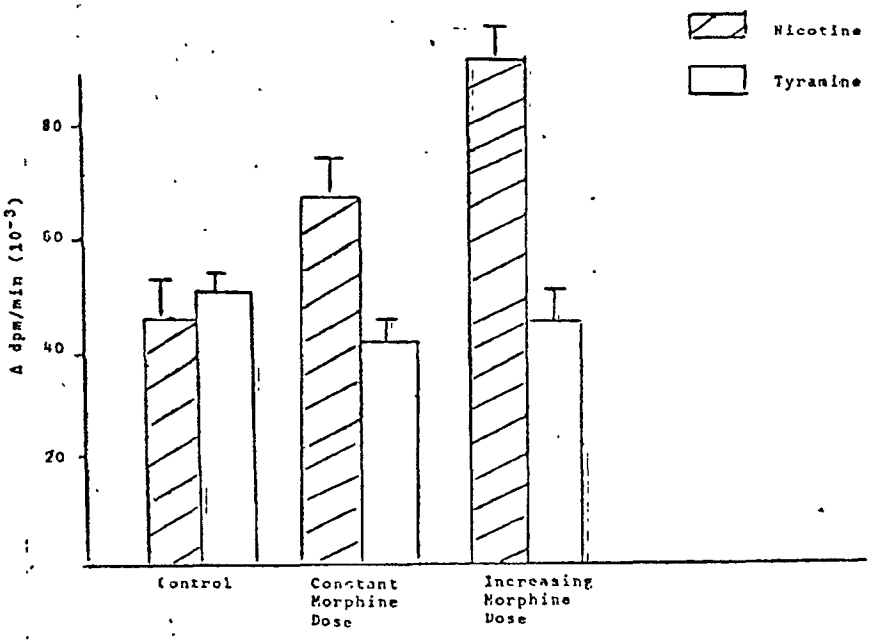

Fig. 8 Depicts the release of ^{3}H-NE from the perfused rabbit heart by nicotine (100 µg) in hearts obtained from control rabbits, rabbits treated with a constant dose of morphine of 15 mg/kg for 5 weeks or increasing doses of morphine up to 90 mg/kg for 5 weeks. Data is plotted as total amount of NE released in dpm/min (10^{-3}) to a 1 min injection of nicotine or tyramine.

In addition to these two observations from our own laboratory with nicotine there are many examples in the literature regarding differences between the acute and chronic administration of other drugs which effect neuronal transmission processes. For instance:

1) The psychoactive drug methamphetamine produces an increase in the concentration of serotonin in most areas of the brain following acute administration, with a decrease in the hypothalamus and cortex. With chronic administration, however, the serotonin content in the caudate nucleus is actually increased--rather than decreased (16).

2) The acute administration of imipramine and protriptylline (2 tricyclic antidepressant drugs) produces a decrease in the turnover of norepinephrine in the brain with no changes in the endogenous content of the amine. The chronic administration of these 2 drugs, on the other hand results in an increase in the turnover of norepinephrine and a decrease in the endogenous norepinephrine content (17).

Another observation made in our laboratory deserves special mention. We have been studying the release of ^{3}H-NE from the perfused rabbit heart in much the same manner as the guinea-pig heart mentioned above. If hearts are obtained from rabbits chronically treated with morphine in a fixed dose of 15 mg/kg/day for 35 days or in gradually increasing doses (up to 90 mg/kg/day) it has been observed that there is a greater release of ^{3}H-NE following nicotine administration in these hearts as compared to controls (Fig. 6). This demonstrates that the chronic administration of this drug (morphine) results in a quantitatively different response of the adrenergic nerve terminals to nicotine.

Since it is very clear that these may be marked quantitative and

qualitative differences between the acute and chronic administration of drugs, such as nicotine, which influence neuronal activity, it would seem of great importance to determine what differences in adrenergic nerve activity might exist between the acute administration of nicotine and following chronic exposure of this agent.

We have a very reproducible measure of the action of nicotine on adrenergic nerve activity that is: a) the release of ^{3}H-NE from the perfused guinea-pig heart and, b) the release of ^{3}H-NE from incubated brain slices. We also have quite a lot of experience in measuring the turnover of neurotransmitters (an in vivo marker for neuronal activity) as well as measurements of metabolic enzyme activity (monoamine oxidase and catechol-o-methyl transferase activity). The purpose of this present proposal therefore, is to study what influence the chronic administration of nicotine to rats and guinea-pigs has on several parameters of neuronal function such as: 1) the release of ^{3}H-NE from the perfused hearts by nicotine (model of peripheral adrenergic synapse). 2) the effect of nicotine on the release of labeled NE, dopamine and serotonin from brain slices obtained from discrete brain regions (model of central symapses) 3) the effect that the chronic exposure of nicotine has on the turnover of NE, dopamine and serotonin (in vivo marker of neuronal activity) and 4) the effect that the chronic exposure of nicotine has on adrenergic metabolic enzyme activity (MAO and COMT activity).

These experiments will enable us to have a good comparison between the acute effect of nicotine on noradrenergic activity and the effect of nicotine on this activity after experimental animals have been exposed to the alkaloid for varying periods of time. This latter situation will more closely mimic what we might expect in

chronic smokers and correlate much better with the effect of smoking on neuronal activity.

B) METHODS OF PROCEDURE

Experiments will be carried out on male rats with initial weights of 150-170 gms and male guinea-pigs with initial weights of 150 gms.

Administration of Nicotine. Animals (rats and guinea-pigs) will be treated with approximately 2.0 mg/kg/day/animal of nicotine alkoloid placed in the drinking water for varying lengths of time. This concentration will be used because it has been shown to be equivalent to the "two-pack-a-day" dose of nicotine (Wenzel et al., 1964, 18-20) and has been shown to produce pharmacological effects. The animals will be caged in groups according to their treatment. Four rats or two guinea-pigs will be placed in each cage. Under these circumstances it has been observed that the animals will receive an average rather than an exact daily dose of 1.0 or 2.0 mg/kg (Wenzel et al. 1964, 18).

Nicotine will be administered in an average concentration of 1.0 or 2.0 mg/100 ml water. The total volume of the nicotine solution to be administered will be kept slighly less than the volume of water which the group will consume in one day. This volume will be given at noon and untreated water will be made available the following morning after the nicotine solution has been consumed. It has been shown that this concentration of nicotine will not impart a taste to the water and the rats show no preference for the solution of nicotine or untreated water. Depending upon the results obtained other doses of nicotine will also be studied.

At varying periods of time of treatment--2 wks., 1, 2, 3, 4, 5, 6, 8, 10, and 12 months--the animals will be killed and hearts and brains removed for determination of the effect of nicotine in releasing

^{3}H-norepinephrine from the perfused heart or brain.

Perfused Heart Preparation. Hearts will be removed from the animals (guinea-pigs and rats) under pentobarbital anesthesia and immediately connected to an Anderson-Craver coronary perfusion apparatus (Metro Scientific Co.) via the aorta. The normal perfusion medium contains in millimoles per liter: NaCl, 119.8; KCl, 5.63; $CaCl_2$, 2.16; $MgCl_2$, 2.10; dextrose, 100 and $NaHCO_3$, 25.0. The solution will be bubbled with 95% O_2 - 5% CO_2; temperature maintained at 37 $\pm$ 1°C and pH at 7.32 to 7.45. All hearts will be perfused at a constant flow of 6.0 $\pm$ .5 ml/min. with a Harvard perfusion pump. Following an equilibration period the hearts will be perfused with 1.0 ng/ml of l-^{3}H-norepinephrine for 20 minutes to label the endogenous store. The hearts will then be switched to a norepinephrine free-medium and the perfusate effluents continuously collected and analyzed. After 10-20 min. of perfusion with a norepinephrine-free medium nicotine in various concentrations will be administered via a side arm cannula.

Analysis of ^{3}H-norepinephrine. The perfusate effluents will be collected in graduated tubes containing ascorbic acid (5 mg). ^{3}H-norepinephrine will then be analyzed by liquid scintillation spectrometer following alumina column chromatography as described in Westfall and Osada (1969, 21) and Westfall and Brasted (1972, 13). For liquid scintillation counting 1.0 ml of sample will be placed in 10 ml of Triton-based solution containing 5.5 g of 2,5-diphenyloxazole (PPO); 150 mg of 1,4-bis [2-(5-Phenyloxazolyl)]-Benzene (POPOP) and 2:1 mixture of toluene and Triton X-100 and counted in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency as determined by external standardization is 18-20%.

Perfused Brain Slices . The rat brain will first be dissected into six regions according to a modification in the procedure described by Iversen and Glowinski (22). These include: medulla-pons; cerebellum; cortex; midbrain, striatium and hypothalamus. The reproducibility of the dissection procedure is depicted on Table 2 which shows the mean weight of each region ± standard error of the mean. The endogenous norepinephrine of each region is depicted on Table 3. These determinations were carried out according to a modification of the automated trihydroxyindole procedure of Robinson and Watts (23). These values agree reasonably well with those obtained from the literature, so also serve as a measure of the reproduciability of the dissection technique.

Following the dissection technique the various brain regions will then be chopped with the McIlwain tissue chopper according to the procedure described by Ziance and Rutledge (24,25). The chopper is set at 0.3 mm and the brain tissue is chopped two times in two directions which are at right angles.

The tissue is then scrapped off the plastic disc into 12 ml centrifuge tubes with Krebs-Henseleit solution. The tissue is thoroughly suspended via a vortex blender. The suspension is then centrifuged in a clinical centrifuge at room temperature for two min. at 1,000 xg. The pellet is then resuspended in physiological salt solution and incubated at 37°C for 10 min. in a shaking water bath. ^{3}H-l-norepinephrine (10 µc; specific activity ~ 6 C/mmol; final concentration of norepinephrine will be 10^{-6}M) will then be added and the tubes incubated for 15 min. at 37° in a 95% O_2 - 5% CO_2 atmosphere. Similar concentration of labeled dopamine and serotonin will be used in experiments studying the release of these neurotransmitters. The total incubation

volume will be 2.0 ml. At the end of the incubation, the samples will be centrifuged as described above, the supernatant is discarded and the pellet resuspended in 3.0 ml of the physiological salt solution by mixing in the centrifuge with a Vortex-Genie mixer. This procedure will be repeated 3 times. The fourth suspension is incubated at 37°C for 20 min. After centrifugation and removal of the medium the tissue is layered on Whatman No. 1 filter paper and placed in a Millipore filter holder jacketed with warm water to maintain temperature. The chopped tissue is then superfused at a constant flow of 0.6 ml/min. by means of a Harvard perfusion pump. The perfusate effluents are collected at 5 min. intervals, separated by alumina column chromatography as described earlier and ^{3}H-norepinephrine, ^{3}H-dopamine, or ^{3}H-serotonin counted by liquid scintillation spectrometer. The tissue will be perfused for approximately 1 hour until the perfusate effluent is very constant, the tissue is then switched to a medium containing nicotine in the presence or absence of various drugs and the perfusate effluent continuously collected and counted.

It has been demonstrated that brain tissue is quite viable following such a procedure and can be used as a valid method for measuring the release of transmitters from neural tissue (26). We have demonstrated that nicotine will release ^{3}H-norepinephrine from various brain regions including hypothalamus, cortex, cerebellum, and medulla-pons using such a technique (Figs. 3-7). The effect of nicotine on the release of dopamine or serotonin is unknown but will be investigated in the proposed study.

REPRODUCIBILITY OF DISSECTION PROCEDURE

Brain Region	Mean Weight mg ± S.E.M.
Medulla - Pons	262 ± 20
Cerebellum	327 ± 31
Cortex	756 ± 49
Midbrain	401 ± 40
Striatum	261 ± 25
Hypothalamus	61 ± 10

Fig. 9

ENDOGENOUS NOREPINEPHRINE CONTENT
OF VARIOUS BRAIN REGIONS

Brain Region	Norepinephrine Content µg/g ± S.E.M.
Medulla - Pons	0.80 ± .21
Cerebellum	0.32 ± .08
Cortex	0.29 ± .09
Midbrain	0.54 ± .09
Striatum	0.53 ± .12
Hypothalamus	1.63 ± .19

Fig. 10

Turnover of Norepinephrine.

At various periods of time of nicotine treatment, measurements of norepinephrine turnover will be made. Animals will be injected with alpha-methyl tyrosine methylester (αMPT) i.v. in a dose of 200 mg/kg followed by a subsequent dose of 100 mg/kg 2 hours later. Animals will be killed at 2, 4, 6 and 8 hours after αMPT and heart and brain removed for extration and analysis of NE. The tissues will be dissected, washed in saline, weighed and homogenized in 5% trichloroacetic acid (heart) or 0.4 N perchloric acid (brain) by an Ultra-Turrax homogenizer. After centrifugation and absorption of the catecholamines on alumina columns, NE will be measured using the automated trihydroxyindole procedure (23). Analysis will be made on the whole heart and discrete brain regions including medulla-pons, striatum, hypothalamus, cerebellum, cortex, and brain stem. These regions will be dissected out according to the procedure described above. Turnover rates will be calculated by multiplying the steady-state level of NE by the fractional rate constant for the decline in endogenous NE after αMPT (27-29)

Monoamine Oxidase Activity.

MAO activity will be carried out according to the method of Wurtman and Axelrod (30). At various periods of time of nicotine administration, animals will be killed by decapitation and 200 mg of liver and 1 whole heart will be taken and homogenized in cold isotonic KCl. The tissue will be homogenized so that the final tissue concentration of liver will be 2 mg/ml. Tissue homogenates will then be incubated in a reaction mixture containing 0.1 M phosphate buffer and

and ^{14}C-tryptamine (New England Nuclear, (S.C. 10 mc/mmole) and the ^{14}C-indoleacetic acid formed extracted into toluene and counted by scintillation spectrometry. Blanks will be prepared by placing them in a boiling water bath for 3 min.

Catechol-o-Methyl Transferase Activity

COMT activity will be measured according to the method of Krakoff et al. (31). One g of tissue (heart or liver) will be homogenized in 4 ml of 1.15% KCl and centrifuged for 10 min. at 10,000 g. An aliquot of the supernatant fraction will then be added to an incubation mixture containing 0.5 M phosphate buffer, 2 M $MgCl_2$, 50 µg epinephrine and 1 µg of 5-adenosyl-L-methionine methyl ^{3}H (New England Nuclear).

SIGNIFICANCE OF THIS PROPOSAL

Nicotine is one of the most important and active ingredients in tobacco. Because of the wide spread use and continual implications of smoking as a health hazard, it is important to have a thorough understanding of the action of this drug on the nervous system. Studies of the acute administration of nicotine have been important in demonstrating the many actions of nicotine. A question of paramount importance however is: TO WHAT EXTENT DOES THE ACUTE ADMINISTRATION OF NICOTINE IN VIVO OR IN VITRO REFLECT THE ADMINISTRATION OF NICOTINE IN HUMANS TAKEN IN THE FORM OF TOBACCO SMOKING? Since smokers are actually chronic users of tobacco, it would appear that studies testing the effect of nicotine on tissues obtained from animals chronically exposed to nicotine would come much closer to mimicing the human situation. This is what we plan to do in this study. We have several very accurate ways of measuring the effect of nicotine on nervous tissue and therefore feel it will be of great importance to make these measurements on tissues taken from animals exposed to nicotine for varying periods of time. We feel this will give us a more accurate picture of what effect nicotine has on nervous tissue of man when it is administered from tobacco smoke. We feel that we will then be able to correlate the effects of nicotine with tobacco smoking in a much more valid way. It appears that up to the present time these studies have not been done so we really don't know what effects nicotine has on synaptic transmission, transmitter turnover, etc. We have reason to suspect that the effects of nicotine on neuronal function might be quite different when studied after animals have been chronically exposed to this alkaloid. We feel, therefore, that the studies suggested in the present proposal are quite important and will provide us with extremely valuable information.

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20. Wenzel, D. G. and Stark, L.G., Am. Heart J. 71:368, 1966.

21. Westfall, T.C. and Osada, H., J. Pharmacol. Exp. Ther. 167:300, 1969.

22. Glowinski, Jr. and Iversen, L. L., J. Neurochem. 13:655, 1969.

23. Robinson, R. L. and Watts, D. T., Clin. Chem. 11:986, 1965.

24. Ziance, R. J. and Rutledge, C.O., J.Pharmacol. Exp. Ther. 180:118, 1972.

25. Ziance, R. J., Azzaro, A.J. and Rutledge, C. O., J. Pharmacol. Exp. Ther. 182:284, 1972.

26. Bollard, B. M. and McIlwain, H., Biochem. J. 66:651, 1957.

27. Brodie, B. B., Costa, E., Dlabac, A., Neff, N.H. and Snookler, H. H., J. Pharmacol. Exp. Ther. 154:493, 1966.

28. Costa, E. and Neff, N. H. in Pharmacology of the Basal Ganglier ed. by E. Costa, pp. 141-155, Rava Press N.Y., 1966.

29. Brodie, B. B. and Reid, W. D., Advan. Pharmacol. 6B:97, 1968.

30. Wurtman, R. J. and Axelrod. J. Biochem. Pharmacol. 12:1439, 1963.

31. Krakoff, L.R., Buccino, R. A., Spann, J.F.,Jr. and deChamplain, J., Am. J. Physiol. 215:549, 1968.

8C. Experience of Principal Investigator

The Principal Investigator has had a wide range of experience in studying the effects of drugs (particularly nicotine-type agents) and physiological manipulations on the synthesis, storage, release, uptake, and metabolism of catecholamines and closely related substances. Therefore, we feel we are well equipped to carry out studies involving the extraction, isolation, separation and measurement of labeled and unlabeled amines and metabolites. These techniques are, in fact, being carried out daily in the Principal Investigator's laboratory. In addition, we have had a lot of experience setting up and conducting studies on isolated and perfused tissue preparations. For these reasons, the Principal Investigator feels that he is well qualified for carrying out the experiments described in this research proposal.

9,10. Facilities Available and Additional Requirements.

These studies will be conducted in Dr. Westfall's laboratory which is housed in the Department of Pharmacology, Jordan Medical Education Building. These are new facilities which we moved into in April, 1972. Office and laboratory space consists of over 800 $ft.^2$. The primary laboratory is well equipped with glassware, ovens, water baths, stirrers, timing devices, etc. The following equipment is available: radiometric pH meter; Beckman pH meter; Packard Tri-Carb (3000 series) Scintillation Spectrometer; Farrand Model A photoelectric fluorometer with various filter combinations; Beckman Model B Spectrophotometer; two Mettler analytical balances; Facit Table Top Calculator; Polytron tissue homogenizers; three Harvard infusion pumps; Brush Mark II electronic recorder, Statham face and pressure transducers; perfusion and isolated tissue chambers; four metabolic and water baths; Technician autoanalyzer for catecholamine determinations.

In addition, other facilities are available which are shared with other Departmental members. These include: four cold rooms; a completely equipped enzyme preparation room; an effective working library with copying facilities; Aminco-Bowman spectrophotofluorometer; two other Technician autoanalyzers; four liquid Scintillation spectrometers; two Gilford 2400 spectrophotometers; automatic diswasher, a range of refrigerated centrifuges including International PR-2, Sorvall RC-2, Beckman ultracentrifuges (L-2, L2-65B, L3-40) with various heads; Olivette Programma 101 computer; ReVCO ultraflow temperature freezer and two computer terminals.

Animals will be maintained under the care of full time veterinarians in the general animal quarters as well as a small animal room located on the same floor as the Department. This will be most convenient in maintaining adequate supervision of the administration of nicotine. The Medical School has a first rate library with over 1600 scientific journals currently on the subscription list.

11. SHORT BIOGRAPHICAL SKETCH OF PRINCIPAL INVESTIGATOR

Estimated percentage of time to be devoted to proposed work - 25%

NAME: Thomas C. Westfall REDACTED

TITLE: Associate Professor of Pharmacology

BIRTHDATE: REDACTED

PLACE OF BIRTH: REDACTED

NATIONALITY: REDACTED

EDUCATION:

West Virginia University, Morgantown, W. Va.,
A.B., 1959, Biology and Chemistry
West Virginia University, Morgantown, W. Va.,
M.S., 1961, Pharmacology
West Virginia University, Morgantown, W. Va.,
Ph.D.,1962, Pharmacology
Karolinska Institute, Stockholm, Sweden, Postdoc.,
1963-64 Neurochemical Pharmacology

HONORS:

Board of Governor Scholarship, West Va. Univ.,1955-59
National Institutes of Health Predoctoral Fellowship,
1959-62
National Institutes of Health Postdoctoral Award
National Heart Institute) 1963-64

PRECEPTORS:

1959-62	Dr. Daniel T. Watts Dean of Graduate Studies Medical College of Virginia Virginia Commonwealth University	Professor U.S.von Euler Chairman of Physiology Karolinska Institute Stockholm, Sweden

SOCIETIES:

REDACTED REDACTED

MAJOR RESEARCH INTEREST:

1003542171

Influence of drugs on uptake, storage, release and inactivation of biogenic amines.

Central and Peripheral Mechanism of action of nicotine; Neuropharmacology; Autonomic Pharmacology

RESEARCH AND PROFESSIONAL EXPERIENCE:

1972-	Director, Medical School Pharmacology Course
1969-	Associate Professor of Pharmacology, Univ. of Virginia School of Medicine
1969-	Chairman, Committee on Graduate Studies and Department Graduate Advisor
1965-69	Assistant Professor of Pharmacology, University of Virginia School of Medicine
1964-65	Assistant Professor of Pharmacology, West Virginia University Medical Center
1963-64	Postdocoral Fellow of National Heart Institute, Department of Physiology, Karolinska Institute, Stockholm, Sweden (Professor U.S. von Euler, Advisor)
1962-63	Instructor in Pharmacology, West Virginia University Medical Center

12. PRINCIPAL PUBLICATIONS DURING THE PAST SEVEN YEARS:

1. Westfall, T. C. Tobacco alkaloids and the release of catecholamines in Tobacco Alkaloids and Related Compounds, Ed. by U.S.von Euler, Pergamon Press, 4: 179, 1965.

2. Westfall, T. C. Effect of nicotine and nicotine analogues on tissue and urinary catecholamines in the rat. Acta Physiol. Scand., 63: 77, 1965.

3. Westfall, T. C. Uptake and exchange of catecholamines in rat tissues after d-and l0 adrenaline. Acta Physiol. Scand., 63: 336, 1965.

4. Westfall, T. C. and Peach, M. J. Action of angiotensim on myocardial and renal catecholamines in the rabbit. Biochem. Pharmacol., 14: 1916, 1965.

5. Westfall, T. C., Cippoloni, B. and Edmundowicz, A. Influence of propranolol on the hemodynamic changes and plasma catecholamine levels following cigarette smoking and nicotine. Proc. Soc. Exp. Biol. Med., 123: 174, 1966.

6. Westfall, T. C., Fleming, R. M., Fudger, M. K. and Clark, W. G. Effect of nicotine and related substances on amine levels in the brain. Ann. N.Y. Acad. Sci., 142: 83, 1967.

7. Westfall, T. C. Accumulation of norepinephrine in rat tissue following treatment with three beta adrenergic antagonists. Arch. Int. Pharmacodyn., 167: 69, 1967.

8. Westfall, T. C. and Anderson, G. P. Influence of nicotine on catecholamine metabolism in the rat. Arch. Int. Pharmacodyn. 169: 421, 1967.

9. Westfall, T. C. Effect of beta adrenergic blockers on the noradrenaline content of rat heart and spleen before and after noradrenaline infusion. European J. Pharmacol., 2: 163, 1968.

10. Westfall, T. C. Action of a beta adrenergic receptor blocking agent on the positive chronotropic response and uptake of norepinephrine in the perfused guinea pig heart. J. Pharmacol. Exp. Ther., 162: 239, 1968.

11. Westfall, T. C. The alpha and beta receptors of the sympathetic nervous system. Va. Medical Monthly, 96: 3, 1969.

12. Dailey, J. W. and Westfall, T. C. Effect of actinomycin D on the recovery of cardiac noradrenaline after depletion with guanethidine. J. Pharma. Pharmacol., 21: 197, 1969.

13. Westfall, T. C. and Osada, H. Influence of adrenalectomy on the synthesis of norepinephrine in the rat heart. J. Pharmacol. Exp. Therap., 167: 300, 1969.

14. Osada, H. and Westfall, T. C. Influence of adrenalectomy on the recovery of noradrenaline levels following guanethidine or metaraminol. Arch. Int. Pharmacodyn., 180: 162, 1969.

15. Westfall, T. C. Effect of alpha-methyl tyrosine on content and subcellular distribution of norepinephrine in rat heart and brain. Life Sciences, 9: 339, 1970.

16. Westfall, T. C. Influence of nicotine administration on blood pressure and turnover of tissue norepinephrine in the rat. European J. Pharmacol., 10: 19, 1970.

17. Brand, E. D. and Westfall, T. C. Neuropharmacology, Chapter 45 in Medical Chemistry, Ed. by Alfred Burger, Third Edition. John Wiley and Sons, Inc., Interscience Publishers, New York, pp. 1190-1234, 1970.

18. Gilmore, J., O'Brien, W., Brand, E. D., Peach, M.J. and Westfall, T. C. A student exercise in clinical pharmacology. Renal effects of diuretics. Clin. Pharmacol. Ther. 12: 759, 1970.

19. Westfall, T. C. and Brase, D. Studies on the mechanism of tolerance to nicotine induced elevations of urinary catecholamines. Biochem. Pharmacol., 20: 1627, 1971.

20. Westfall, T. C. Nervous system stimulants in Educational Perspectives on the Drug Crisis. Ed. by P. Hackett, W. M. Lewis, and J. B. Pierce, Jarmen Press, 1971.

21. Peach, M. J. and Westfall, T. C. Potentiation of adrenal medullary responses to angiotensin by [4,4'-biphenylenebis-(2-Oxoethylene)] Bis [(2,2-Diethoxyethyl)-Dimethylammonium Bromide] (DMAE) in Vitro. J. Pharmacol. Exp. Ther. 181: 422, 1972.

22. Westfall, T. C. and Brasted, M. Mechanism of action of nicotine on adrenergic neurons in the perfused guinea-pig heart. J. Pharmacol. Exp. Ther. 182: 409, 1972.

23. Brase, D. A. and Westfall, T. C. Stimulation of rat liver phenylalanine hydroxylase activities by derivatives of Vitamin E. Biochim. Biophy. Res. Comm. 48: 1185, 1972.

24. Westfall, T. C. and Brasted M. Effect of 4,4' Biphenylenebis-[(2-oxoethylene-Bis-(2,2 Diethoxyethyl)] dimethylammonium Bromide (DMAE) on the uptake and nicotine induced release of norepinephrine in the heart. J. Pharmacol. Exp. Ther. 184:198, 1973.

PAPERS IN PRESS OR IN PREPARATION:

Dailey, J. W. and Westfall, T. C. The effects of adrenalectomy and adrenal steroids on the synthesis of norepinephrine in the rat. J. Pharmacol. Exp. Ther. (In Press) 1973.

Westfall, T. C. and Peach, M. J. Influence of equilibrium perfusion duration on H^3-norepinephrine uptake, myocardial pacemaker sensitivity and intracellular cation concentrations in isolated guinea pig hearts. Proc. Soc. Exp. Biol. Med. 142: (Jan.), 1973.

Westfall, T. C. and Lewis, T. C. Effect of aminoglutethamide on norepinephrine turnover in the rat heart. Proc. Soc. Exp. Biol. Med.

Atuk, N. O., Westfall, T. C. and Westfall, V. Altered catecholamine metabolism in recurrent juardice evidence for catechol-o-methyl transferase deficiency.

Brase, D. A. and Westfall, T. C. Studies on the mechanism of stimulation of phenylalanine hydroxylase activity by short chain alcohols. Biochem. Biophy. Acta.

Hsu, C-Y and Westfall, T. C. Release of ^{3}H-norepinephrine by aminophylline in the perfused guinea-pig heart.

Westfall, T. C. and Brasted, M. Specificity of blockade of the nicotine-induced release of norepinephrine from adrenergic neurons by various pharmacological agents.

PUBLISHED ABSTRACTS DURING THE PAST SEVEN YEARS:

1. Peach, M. J. and Westfall, T. C. Action of angiotensin on myocardial catecholamines in the rabbit. Fed. Proc., 24: 488, 1965.

2. Westfall, T. C. Influence of pronethalol, propranolol and iproveratril on uptake and storage of norepinephrine. Fed. Proc., 25: 260, 1966.

3. Westfall, T. C., Fleming, R. M., Fudger, M. K., and Clark, W. G. Effect of nicotine and related substances on amine levels in the brain. Symposium on The Effect of Nicotine and Smoking on the Central Nervous System. N.Y. Acad. Sci., April, 1966.

4. Westfall, T. C. Uptake and storage of norepinephrine following the administration of three beta adrenergic antagonists. Va. J. Sci., 17: 354, 1966.

5. Westfall, T. C. Influence of nicotine on catecholamine metabolism. Symposium Abstract. Tobacco and Health. Amer. Med. Assn., November, 1966.

6. Westfall, T. C. Influence of beta adrenergic antagonists on norepinephrine content in rat heart before and after NE infusion. Fed. Pro., 26: 569, 1967.

7. Westfall, T. C. Influence of beta adrenergic blockers on norepinephrine storage in the perfused guinea pig-heart. Va. J. Sci., 18: 202, 1967.

8. Westfall, T. C. The effect of beta adrenergic blocking drugs on the norepinephrine level in the perfused guinea-pig heart following NE infusion. The Pharmacologist, 9: 249, 1967.

9. Westfall, T. C. and Osada, H. Influence of adrenalectomy on the depletion of norepinephrine following treatment with metaraminol or guanethidine. Fed. Proc., 27: 601, 1968.

10. Moore, W. C. and Westfall, T. C. The influence of monoamine oxidase inhibitors on the accumulation of norepinephrine in reserpine treated rats. Va. J. Sci., 19: 206, 1968.

11. Westfall, T. C. and Osada, H. Influence of adrenalectomy on the turnover of norepinephrine in the rat heart. The Pharmacologist, 10: 158, 1968.

12. Westfall, T. C. Influence of nicotine on the turnover of norepinephrine in brain and heart. J. Amer. Med. Assn., 1968.

13. Atuk, N. O., Westfall, T. C. and Donaldson, M. H. Catecholamine metabolism and alpha-receptor response to norepinephrine in familial pheochromocytoma. Clin.Res., 17: 57, 1969.

14. Westfall, T. C. and Brase, D. A. Adrenal stimulation and monoamine oxidase activity following daily nicotine treatment. Fed. Proc., 28: 287, 1969.

15. Atuk, N. O., Westfall, T. C. and Donaldson, M. H. Mechanism of normal blood pressure in familial pheochromocytoma. Proceedings of Am. Coll. of Physicians, 129-130, 1969.

16. Westfall, T. C. The effect of nicotine on the synthesis, uptake and metabolism of catecholamines. Proceedings of the Fourth International Pharmacol. Congress, Basel. July: 153, 1969.

17. Colombini, C., Westfall, T. C., and McCoy, E. The effect of LSD-25 on vitamin B-6 metabolism and brain amine levels in mice. Proc. Second International Meeting of Neurochem., Milan: 133, 1969.

18. Colombini, C., Westfall, T. C. and McCoy, E. Effects of LSD-25 and marijuana on vitamin B-6 synthesis and distribution in the mouse. Proc. Southeastern Sect. Amer. Chem. Soc., 1969.

19. Dailey, J. W. and Westfall, T. C. Effect of adrenal steroids on the turnover of H^3-norepinephrine in the rat heart. Fed. Proc., 29: 413, 1970.

20. Westfall, T. C. and Peach, M. J. Influence of equilibration perfusion duration on H^3-norepinephrine uptake and intracellular cation concentration in isolated guinea-pig hearts. Pharmacologist, 12: 234, 1970.

21. Dailey, J. W. and Westfall, T. C. Influence of adrenalectomy and steroid replacement on norepinephrine biosynthesis. Va. J. Sci., 21: 144, 1970.

22. Colombini, C., Westfall, T. C. and McCoy, E. The changes in Vitamin B-6 and brain amine metabolism in mice chronically treated with 9-tetrahydrocannabinol. Proceed. of International Psychopharmacol. Congress. Prague, August, 1970.

23. Atuk, N. O. and Westfall, T. C. The occurence of hypertension in benign recurrent interhepatic cholestases. Clin. Res., 19: 80, 1971.

24. Westfall, T. C. Interaction of nicotinic and antinicotinic agents on heart rate and uptake of norepinephrine. Fed. Proc., 30: 446, 1971.

25. Brase, D. A. and Westfall, T. C. Stimulation of phenylalanine hydroxylase activity by short chain alcohols. Pharmacologist, 13: 193, 1971.

26. Westfall, T. C. Studies on the mechanism of nicotinic agents on adrenergic nerve terminals. Pharmacologist, 13: 229, 1971.

27. Westfall, T. C. Action of beta-adrenergic receptor blocking agents on the turnover of norepinephrime in heart and brain. Fed. Proc. 31: 567, 1972.

28. Atuk, N. O. and Westfall, T. C. Reduced catechol-o-methyl transferase activity in the liver and increased pressor response to norepinephrine. Am. Soc. Clin. Invest. 55, 1972.

29. Westfall, T. C. Further studies on the mechanism of norepinephrine release by nicotine in the perfused guinea-pig heart. Proceed. Fifth Internat. Congress on Pharmacology, 1972, San Francisco.

13 Budget: (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Thomas C. Westfall	25%	REDACTED
Technical		
Lab Specialist A (Mary Brasted)	100%	REDACTED
	Sub-Total	REDACTED
B. Consumable Supplies (list by categories)		
Chemicals and Isotopes		1,500.
Animals and Animal Care		5,000.
	Sub-Total	6,500.
C. Other Expenses (itemize)		
Publications (400.)		
Travel (300.)		
Computer time(500.)		1,200.
	Sub-Total	1,200.
D. Permanent Equipment (itemize)		
Tissue Slicer		600.
		1003542178
	Sub-Total	600.
E. Overhead (15% of A+B+C)		2,785.
	Total	REDACTED

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	6,500	1,400	0	2,925	REDACTED
Year 3						

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature Thomas C Westfall
Director of Project

Telephone

Signature Ray C. Gardner
Business Officer of the Institution

Telephone

JUSTIFICATION OF BUDGET

1. Personnel

Principal Investigator. Dr. T.C. Westfall has had considerable experience in the field of neurotransmitter synthesis, storage, release and metabolism and in conducting experiments at the tissue and biochemical level. He will direct and coordinate the various phases of the proposed research and will dedicate about 25% of his time to it. The salary support requested for Dr. T.C.Westfall is less than the amount represented by his per cent of effort to be devoted to this project. The differences will be applied to the University Cost Sharing Commitment.

Laboratory Specialist. The salary requested is for Miss Mary Brasted who has been working in Dr. Westfall's laboratory for three years. She is very experienced and extremely competent in conducting studies on perfused organs and isolated tissues. She will be responsible for treating the animals and in carrying out the various measurements as described in the methods.

2. Equipment

Only one piece of permanent equipment is being requested, that of a MacIlwain tissue slicer. This item is necessary to perpare all the brain slices. We are currently borrowing such an instrument.

3. Supplies

This constitutes the other major individual item in the budget and includes animal costs and care, chemicals, isotopes and glassware.

a) Animals and Animal Care. This item is necessary because of the anticipated and calculated cost of the large numbers of rats and guinea-pigs which will be used to successfully complete this project. Guinea-pig cost $5-6.00 each, $0.12/day for care and rats cost $3.00 each, $0.06/day for care.

b) Chemicals, Isotopes. The biggest item here will be the cost of isotopes, which will be used for the project, including l-^{3}H-norepinephrine $120.00/1 mCi; ^{3}H-dopamine, $70/1 mCi; ^{3}H-serotonin $105.00/1 mCi. In addition there will be a fairly large amount of chemicals necessary.

4. Travel

This item of the budget will enable the Principal Investigator to attend one meeting of the Pharmacological Society.

5. Other Expenses

These include a request for publication costs and commuter time.

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

	Title of Project	Source	Amount	Duration
Current	Role of Cholinergic Agents in Adrenergic Transmission	National Institutes of Neurological Diseases and Stroke	62,970	Two yrs. 6-1-72 5-31-74
Pending	None			

FROM WALTER B. ESSMAN, M.D., Ph.D.
Re Grant Application #467C
May 21, 1973

RESEARCH PROPOSAL EVALUATION
Dr. Thomas C. Westfall

Action of Nicotine on Peripheral and
Central Neurons In Animals Chronically Exposed to Nicotine

The proposal is a well-organized, carefully conceived series of studies which have been developed logically from previous experiments originating in the investigator's laboratory. It would seem that many of the techniques proposed for use in the investigation have been carefully developed and well utilized. The proposed use, particularly of regional tissue from the central nervous system, is interesting and the use of the perfused heart preparation is also most appropriate. I believe that the proposal generally carefully considered some of the important aspects of nicotine action and are quite sound both methodologically and theoretically. One point made in the proposal that I would take some exception to is the ready willingness of the investigator to correlate the effects of nicotine in these proposed experiments with the effects of tobacco smoking in man. I doubt that this could or even should be considered, but I do not think that it detracts appreciably from the quality of the proposed experiments or their significance. I believe that the proposal in general is quite good and that the investigator has presented an impressive array of experiments which will yeild reasonable results.